AD_____

Award Number: W81XWH-06-1-0711

TITLE: Novel Serum Inflammatory Biomarkers for Early Detection of Breast Cancer

PRINCIPAL INVESTIGATOR: Thomas R. Brown, Ph.D.

CONTRACTING ORGANIZATION: Oxford Biomedical Research, Inc.
Rochester Hills, MI 48309

REPORT DATE: June 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-06-2008	Final	1 SEP 2006 - 28 MAY 2008

4. TITLE AND SUBTITLE

Novel Serum Inflammatory Biomarkers for Early Detection of Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0711

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Thomas R. Brown, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Oxford Biomedical Research
Rochester Hills, MI 48309

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Proteins that are post-translationally modified by reactive oxygen and nitrogen species (ROS/RNS) have relevance to disease including cancer however, their study as a source of cancer biomarkers is still at a relatively early stage. Identifying these biomarkers in serum presents a difficult task given the vast range of protein concentrations. To alleviate this problem two enrichment strategies that precede analysis are emerging. The first is removal of high abundance proteins and the second, enrichment of the adducted proteins using immunopurification with antibodies developed against specific protein adducts. Using the latter approach, we isolated and identified four NT-containing proteins unique to the serum of breast cancer patients: obscurin, nucleoprotein, fibrinogen gar and fibrinogen bet. The potential impact of this work is that first, it provides a methodological template for the purification and further study of post-translationally-modified proteins and secondly, through the development of assays to monitor these biomarkers in the serum of cancer patients, it has the potential to present the physician an opportunity for more timely therapeutic intervention by providing improved diagnostic capability with use of early disease biomarkers. This should result in improving long term survival which is highly relevant to the goals of the Era of Hope.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	10	

Table of Contents

Page

Inflammatory Biomarkers For Early Detection Of Breast Cancer

Introduction:
Proteins that are post-translationally modified by reactive oxygen and nitrogen species (ROS/RNS) have relevance to disease including cancer however, their study as a source of cancer biomarkers is still at a relatively early stage (1). Identifying these biomarkers in serum presents a difficult task given the vast range of protein concentrations. To alleviate this problem two enrichment strategies that precede analysis are emerging. The first is removal of high abundance proteins and the second, enrichment of the adducted proteins using immunopurification with antibodies developed against specific protein adducts. Here we design and examine a new modification of the latter approach by purifying nitrotyrosine adducted proteins, the serum nitroproteome, from patients with ductal adenocarcinoma.

Body:

Although recent advances in the early diagnosis of breast cancer have been beneficial, 34% of patients have regional or distal spread of their disease at the time of diagnosis. This contributes to high mortality rates and suggests that further improvement in early diagnosis is urgently needed (1).

A broad range of approaches to develop biomarkers that are capable of detecting early disease or, in some instances, risk of disease are becoming available. DNA methodology includes copy-number assessment, mutation screening, and expression profiles. Additional methodologies include metabolomics, glycomics, and proteomics (1).

There is a growing interest in post-translational modifications (PTMs) of proteins as a source of potential biomarkers for cancer. PTMs, including altered glycosylation, and other oxidative/nitrative (ROS/RNS) stress-induced changes in proteins, are important in many biological processes and have relevance to disease including cancer (1).

Chronic inflammation associated with ROS/RNS at the site of early tumorigenesis has been demonstrated in several studies (2,3). In breast cancer, chronic inflammatory reactions set the stage for initiation of carcinogenesis. This inflammation results in a prolonged release of highly reactive oxidants that may damage the genomes of nearby cells and increase their rate of mutation. In addition to this direct oncogenic effect, a chronically inflamed microenvironment possesses many mechanisms with which to promote progression of a preneoplastic lesion (4). RNS and peroxynitrite rapidly nitrate tyrosine forming to form 3-nitrotyrosine (3-NT) found to be associated with over fifty disease states including various cancers (3). Collectively, these studies suggest that tumor-related modified proteins, including those adducted with 3-NT, may be produced in cancer cells during the early stages of tumor development, and released to the circulation. As such, they may serve as biomarkers for early disease.

Here we have used anti 3-NT immunopurification and examined 3-NT-adducted, tumor-related proteins in the sera of patients with ductal adenocarcinoma. Two methods of protein identification were evaluated following immunopurification of patient's serum. First, the proteins of interest were separated by PAGE and in-gel digestion was performed followed by MS. Secondly, proteins were digested and LC/MS was used for purification and identification. The findings of this study will be used to realize our long term goal which is to develop a panel of validated biomarker immunoassays that detect early or pre-clinical breast cancer.

Antibody production: Antisera against 3-nitrotyrosine (3-NT) was produced in goats immunized with nitrated KLH. The serum was fractionated with 33% saturated ammonium sulfate, dialyzed against 0.01M PBS and stored at -80^{o}C until use.

Affinity purification of NT antibodies: A NT-containing peptide, YPYDVPD-NT-AGGGK, was coupled to cyanogen bromide activated Sepharose Fast Flow at concentrations of 1.5 to 2 mg/ml resin according to the manufacture's instruction (Sigma/Aldrich). Antibody aliquots of 3 mL were diluted 1:4 with PBS and applied to 1 mL of resin. Various flow rates were used during the application of the antiserum to the resin and with the subsequent washes before elution. The resin was washed with 10 column volumes of 0.01M PBS with a 2 min wait for each mL of wash buffer. The antibodies were eluted with 0.1M glycine, pH 2.3. 1.4 mL of eluate was collected in tubes containing 600 µL 2M Tris buffer, pH 8.0 (collection buffer) and mixed. Tubes 1 – 4 were pooled and dialyzed against PBS. The immunoglobulin was precipitated with acetone or buffer exchanged and concentrated by centrifuge filtration (Millipore Ultra 15). Following elution, the resin was immediately washed with 10 mL of collection buffer followed by 15 mL of 0.01M PBS, pH 7.4 containing NaN_3 and stored at 4 ^{o} C until use.

NT affinity chromatography: NT affinity resin was prepared as above using 2 mg affinity purified NT antibody/mL resin.

Nitration of proteins: Various proteins were nitrated using the method of Sokolovsky (6).

Creation of standard serum sets: Blood was collected from patients diagnosed with breast ductal adenocarcinoma. Serum specimens, collected for a separate study, were coded and stored at –80C until use. Age matched controls were purchased from Innovative Research, Novi, MI. Serum aliquots, from both patient groups, were pooled to produce standard serum sets for screening. Each of the six pools contained aliquots from 3 patients and a total of 150 uL serum (7).

Affinity purification of NT-containing proteins in patient's sera.
Each serum pool (150 to 300 uL) was purified by a NT affinity column following the general details outlined above. Following elution, the NT-containing serum proteins were dialyzed against water, precipitated with acetone or buffer exchanged and concentrated by centrifuge filtration (Millipore Ultra 15).

PAGE 8 - 16% Tris glycine gradient gels were obtained from Invitrogen. Purified and concentrated NT-containing proteins and controls were resolved according to the manufacturer's instructions.

Proteomics:
All proteomics analysis was performed at the Proteomics Shared Facilities, Oregon Health & Science University.

Competitive ELISA for the determination of NT antibody specificity.
A 13 mer peptide, **NT**PYDVPDYAGGGK was synthesized by Celtek Bioscience, LLC, (Nashville, TN). A 33 mer peptide, **NT**-YYA-**NT**-YYA-**NT**-YAGPSLKLLSIKGVIVHRLEGVE, was synthesized by Peptide Technologies Corp. (Gaithersburg, MD). The ELISA was established by diluting the 13 mer NT containing peptide to 0.5 ug/mL with 0.1 M Na2CO3, pH 9.6, and adding 200 uL/well of the solution to 96 well plates. The plates were incubated at 4^{o}C overnight and washed 3 times with wash buffer. They were blocked with a 1:10 dilution of gelatin in PBS (fish skin gelatin, Sigma-Aldrich, St Louis, MO) for 1 hour, washed three times with wash buffer, dried overnight in a desiccator at room temperature and stored dry at 4^{o}C until use. Free NT, chlorotyrosine and tyrosine were purchased from Sigma-Aldrich. All ELISA competitors were diluted in assay buffer, 0.01M PBS containing 0.1% gelatin (Bio-Rad Laboratories, Richmond, CA), antibodies against 3-NT were produced in goats, fractionated in ammonum sulfate and diluted in assay buffer .

FIGURE 1 Characterization of the NT antibody:

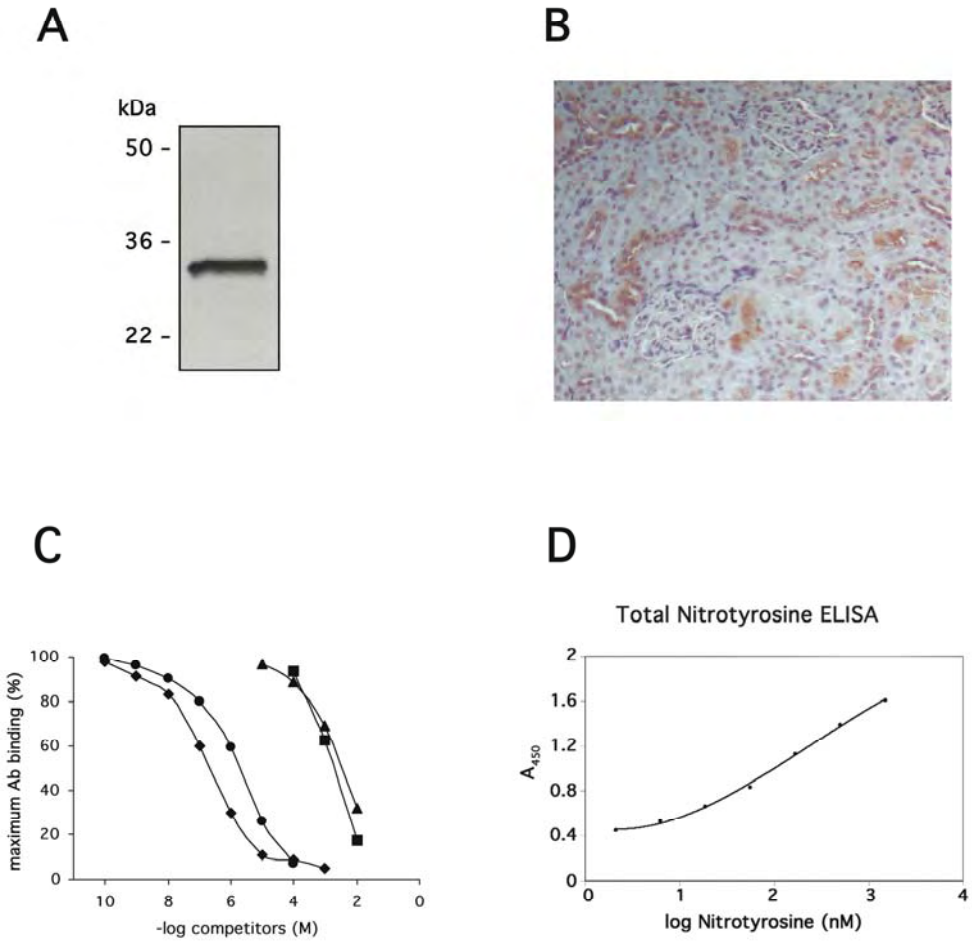
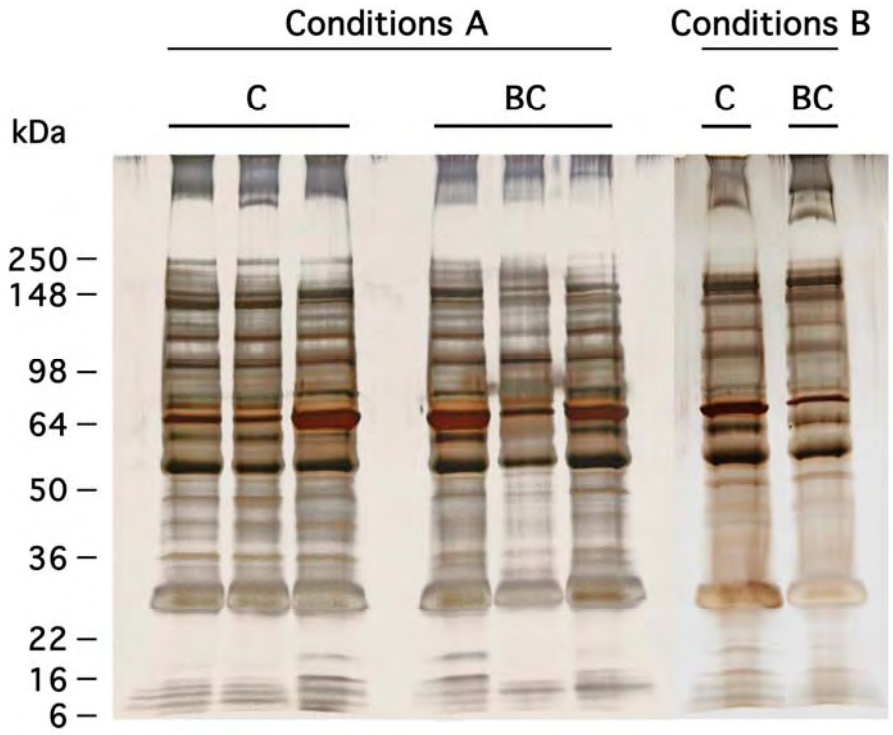


Fig 1(A) Carbonic anhydrase was nitrated, resolved on a 4-20% gradient PAGE, transferred to a NC membrane and stained with affinity purified NT antibody at a concentration of 1:5000, followed by incubation with anti goat IgG coupled to alkaline phosphatase. A single band was present near the 36K MW standard. (**B)** Section of a kidney from a rat treated with N-3,5-dichlorophenyl succinimide (NDPS). The tissue was incubated anti NT antibody at a concentration of 1:5000. Tubules, but not glomeruli, were stained positive for the presence of 3-NT. (**C)** Nitrotyrosine antibody specificity. Competition for NT antibody binding to a NT containing 13 mer peptide coated on a 96 well plate. ELISA competitors are free nitrotyrosine (◆), NT-containing 33 mer peptide (●□, chlorotyrosine (■□, and tyrosine (σ)□□Competition for Ab binding was most effective with free NT followed closely by the NT containing 33 mer peptide. This suggests that the Ab will recognize free NT as well as NT containing proteins in biological fluids. Significant competition by tyrosine and chlorotyrosine occured at > two orders of magnitude higher concentrations of each indicating very good NT Ab specificity. (**D)**
A standard curve from a competitive 3-NT ELISA.

We affinity purified NT containing proteins before tryptic digestion unlike Rush who performed a tryptic digest before immunoprecipitation (5).

Keratin and contamination, a ubiquitous problem in proteomics studies, proved to be a major consideration in the study. Significant amounts of keratin was present in the original specimens. Care was taken after we received the specimen to prevent further keratin contamination.

Figure 2 The presence of non-specific binding of high abundance serum proteins in NT affinity resins.



Affinity resin	Conditions A	Conditions B
Resin volumn	1 mL	0.1 mL
Pretreat resin	none	4.0% Tween
Serum composition	Pool of 3 patients/lane	Pool of 3 patients/lane
Serum applied	300 uL	1.0 mL
% eluate/gel lane	100%	100

Fig. 2 shows PAGE patterns of NT affinity column eluates from breast cancer patients (BC) and controls (C). Each lane is a pool of 3 specimens. The standard immunoprecipitation step (Conditions A) resulted in the co-elution of high abudance serum proteins that bound to the resin non-specifically. In an attempt to decrease NSB, Conditions B were employed (table) and while they diminished NSB somewhat, it still remained an issue. Thus we strongly recommend the removal of high abundance of proteins before the NT affinity step is performed.

FIGURE 2 Tandem mass spectrum of a NT-containing peptide 13 mer peptide.

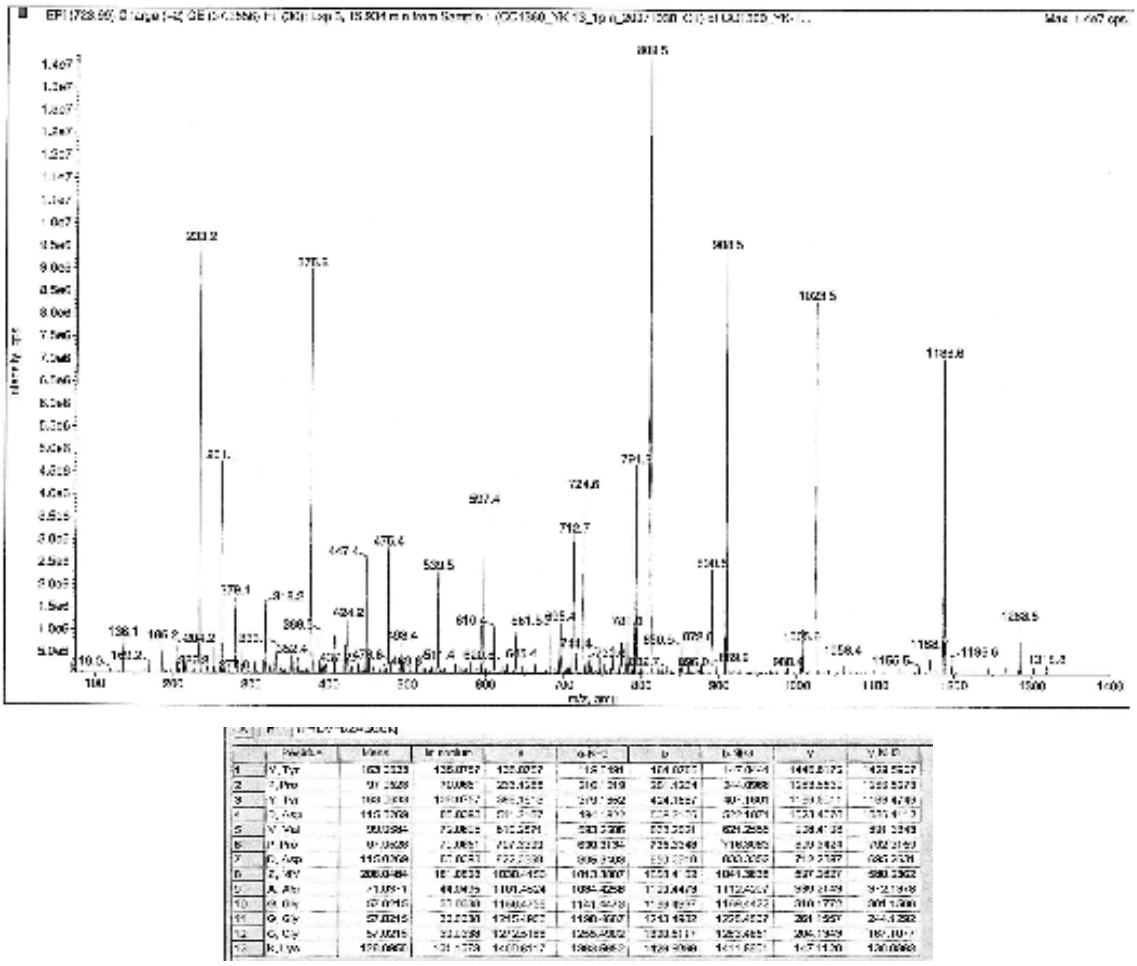


Fig 3. We synthesized a 13 mer, NT containing peptide (YPYDYPD-NT-AGGGK) and examined it by tandem mass spec to identify its sequence. The MS/MS mass spectrum clearly demonstrates the detection and location of the nitrated tyrosine. The peptide gives a strong signal by EMS and a strong y ion series in the MS/MS fragmentation patterns thus validating this methodology for the identification of NT containing peptides.

Using LC/MS and immunoaffinity anti NT affinity resins, we have identified 4 potential breast cancer biomarkers of interest, all of which are present in the cancer patients and absent in the controls: obscurin, nucleoprotein, fibrinogen gar and fibrinogen bet.

Key research Accomplishments

- Described and applied a new methodological approach to the study of oxidation/inflammation-associated post-translationally adducted proteins.
- Identified 4 potential breast cancer biomarkers of interest, all of which are present in the cancer patients and absent in the controls: obscurin, nucleoprotein, fibrinogen gar and fibrinogen bet.

Reportable Outcomes

Poster presentation, Era of Hope meeting for DOD, BCRP, 6/25-28, 2008, Baltimore, Maryland

Conclusions:

We have developed a unique approach to identify potential biomarkers of early breast cancer by enriching breast cancer patient's sera for nitrotyrosine containing proteins, products of nitrosative stress and markers of chronic inflammation, often found in cancers (2). Using this approach we isolated and identified four NT-containing proteins unique to the

serum of breast cancer patients: obscurin, nucleoprotein, fibrinogen gar and fibrinogen bet. The potential impact of this work is that it provides a methodological template for the purification and further study of post-translationally-modified proteins and through the development of assays to monitor these biomarkers in the serum of cancer patients, it has the potential to present the physician an opportunity for more timely therapeutic intervention by providing improved diagnostic capability with use of early disease biomarkers. This should result in improving long term survival which is highly relevant to the goals of the Era of Hope.

References:

1. Hanash, SM, et. al. Mining the plasma proteome for cancer biomarkers. Nature 452:571-579, 2008.

2. Swann, JB, et.al. Demonstration of inflammation-induced cancer and cancer immunoediting during primary tumorigenesis. PNAS 105(2):652-656, 2008.

3. Federico, A, et.al. Chronic inflammation and oxidative stress in human carcinogenesis. Int J Cancer. 121(11):2381-2386, 2007.

4. Calogero RA, et.al. Inflammation and breast cancer. Inflammatory component of mammary carcinogenesis in ErbB2 transgenic mice. Breast Cancer Research 9:211-221, 2007.

5. Rush, J, et. al. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nature Biotech. 23 (1):94–101, 2005.

6. Sokolovsky, M, et al. Tetranitromethane. A Reagent for the Nitration of Tyrosyl Residues in Proteins. Biochemistry 5 (11):3582, 1966

7. Skates, SJ, et al. Pooling of case specimens to create standard serum sets for screening cancer biomarkers. Cancer Epidemiol Biomarkers Prev. 16(2), 334-341, 2007

Appendices - none